Prolactin maintains transient melaninconcentrating hormone expression in the medial preoptic area during established lactation

TITLE:

Prolactin maintains transient MCH expression in the mPOA during established lactation

AUTHORS:

IC Kokay[1], DR Grattan[1], JF Murray[2]

ADDRESSES:

1. Centre for Neuroendocrinology, Department of Anatomy, School of Biomedical Sciences, University of Otago, Dunedin, New Zealand

2. Faculty of Science and Technology, University of Westminster, London, UK and now Centre for Discovery Brain Science, University of Edinburgh, Edinburgh, UK.

Email: jmurra19@exseed.ed.ac.uk

KEYWORDS: melanin concentrating hormone, prolactin, pSTAT5, medial preoptic area, late lactation

The authors of the manuscript have no conflicts of interest to declare.

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abstract

A population of neurones in the medial part of the medial preoptic area (mPOA) transiently express melanin concentrating hormone (MCH) in mid to late lactation in the rat, and this expression disappears on weaning. Prolactin is known to mediate many of the physiological adaptations that occur within the dam associated with lactation and the mPOA is well endowed with prolactin receptors (Prlr) hence we hypothesized that these transiently MCH-expressing cells may be regulated by prolactin. By *in situ* hybridization we show that approximately 60 % of the cells expressing prepro-MCH (*Pmch*) mRNA in the medial part of the mPOA on Day 19 of lactation also express *Prlr* mRNA. To demonstrate that these transiently MCH-expressing cells can acutely respond to prolactin, , dams were treated with bromocriptine on the morning of Day 19 of lactation and then given vehicle or prolactin 4 h later. In the prolactin-treated animals, over 80 % of the MCH-immunopositive cells were also immunopositive for phosphorylated signal transducer and activator of transcription 5 (pSTAT5), an indicator of prolactin receptor activation: double immunopositive cells were rare in vehicle-treated animals. Finally, the effect of manipulating the circulating concentrations of prolactin on Days 17, 18 and 19 on the number of MCH-immunopositive cells on Day19 was determined. Reducing circulating concentrations of prolactin over Days 17, 18 and 19 of lactation with or without a suckling stimulus resulted in a reduction ($p < 0.05$) in the number of MCH-immunopositive cells in the medial part of the mPOA on Day 19 of lactation. Further research is required to determine the functional role(s) of these prolactin-activated transiently MCH-expressing neurones

however we suggest the most likely role involves adaptations in maternal metabolism to support the final week of lactation.

285 words

Introduction

Melanin concentrating hormone (MCH) is a neuropeptide predominately expressed in the incerto-hypothalamus and lateral hypothalamus and has roles in a wide variety of physiological functions including energy balance and reproduction (reviewed by both: 1, 2). Additional expression of prepro-MCH (*Pmch*) in the medial part of the medial preoptic area (mPOA) and the paraventricular nucleus (PVN) during lactation was first described by Knollema and colleagues (3). In virgin or pregnant rats, *Pmch* expression is not detected in these regions (3, 4). The *Pmch*/ MCH-immunopositive cells first appear in the medial part of the mPOA and PVN in mid-lactation (Days 8-14) in the rat and the highest number have been reported on Days 15-21 (3, 4). There is no MCH-immunopositive staining in these cell bodies after weaning (3). A decrease in the number of MCH-immunopositive cells within the mPOA between Days 15 and 21 of lactation independent of the suckling stimulus has been reported (5). However others have reported that the number of suckling pups was positively correlated with the number of MCH-immunopositive cells within the mPOA on Days 12, 15 and 19 of lactation and that the number of cells increased as lactation progressed (6).

The mPOA of the lactating dam is associated with changes in maternal behaviour (7, 8, 9; reviewed by 10, 11, 12, 13). To date however the emphasis has been on understanding the role of the mPOA in establishing both maternal behaviours and maternal physiological responses to the initiation and maintenance of early lactation. There is a paucity of research investigating the period of late lactation

even though it is recognised that there are changes in maternal behaviour (14) as well as changes in both the dam's food intake and body weight (15) and, if she is not already pregnant, the activity of her reproductive axis in anticipation of oestrus following weaning (16). The function of the cells transiently expressing MCH in the medial part of the mPOA are unknown but it is not unreasonable to speculate that they may be involved in one or more of these changes in maternal behaviour and physiology in late lactation. The role of prolactin in stimulating the initiation of maternal behaviours during lactation is well established (reviewed by 10, 13, 17). The mPOA is a brain region rich in prolactin receptors (Prlr: 13, 18, 19, 20, 21) and the number of receptors increases with lactation (22, 23). Prolactin concentrations remain high through lactation, stimulated by the suckling stimulus (25, 26). We therefore hypothesized that the transient expression of MCH in the medial part of the mPOA in late lactation is regulated by prolactin.

Whilst transient expression of MCH has been reported in cells in both the mPOA and the PVN, in the present study we have exclusively investigated the population found in the medial part of the mPOA. The first aim of this study, was to determine if *Pmch* and *Prlr* co-express in the mPOA on Day 19 of lactation. Having demonstrated co-expression, the second aim was to demonstrate if these MCH-immunopositive cells on Day 19 of lactation would acutely respond to prolactin by expressing immunoreactive phosphorylated signal transducer and activator of transcription 5 (pSTAT5), an indicator of prolactin receptor activation. The majority of the MCH-immunopositive cells in the mPOA did acutely respond to prolactin. Finally, the

92 effect of reducing circulating concentrations of prolactin in late lactation on the
93 number of MCH-immunopositive cells within the medial part of the mPOA on Day 19
94 of lactation was determined. Two methods of reducing circulating prolactin
95 concentrations were employed to control for any suckling stimulus effects. Lactating
96 rats suckling 8 pups were administered with a dopaminergic agonist, bromocriptine,
97 to inhibit prolactin release on Days 17, 18 and 19. The suckling stimulus was
98 maintained in these animals through continuous cross-fostering. Another group of
99 lactating rats suckling 8 pups had their pups removed on Day 17 resulting in both a
100 decrease in prolactin release and the removal of the suckling stimulus.

101

102 **Methods**

103 *Animals and experimental treatments*

104 Female Sprague-Dawley rats aged 10 weeks were obtained from the Hercus Taieri
105 Research Facility at the University of Otago. Animals were group-housed (n=6 per
106 cage), unless stated otherwise, and maintained under a 14:10 h light:dark cycle with
107 an ambient temperature of 22 ± 1°C. Food and water were available *ad libitum*
108 throughout the duration of the experiments. All experimental procedures were
109 approved by the University of Otago Animal Ethics Committee.

110

111 To generate lactating rats, the stage of the oestrous cycle was monitored daily by
112 collection of vaginal smears and on the day of a positive proestrous smear, individual
113 rats were housed overnight with a male and mating confirmed by the presence of

114 spermatozoa in the vaginal smear the following morning. Pregnant females were
115 individually housed from around day 16 of pregnancy. On Day 2 postpartum (day of
116 birth=Day 0 postpartum), litters were normalized to 8 pups each and then circulating
117 prolactin concentrations were manipulated on Days 17-19 of established lactation as
118 described next.

119

120 One group of lactating rats (vehicle plus suckling, n=6) received vehicle (250 µl saline
121 in 10% ethanol) sub-cutaneously at 8 am and 6 pm on Days 17 and 18 of lactation
122 and at 8 am on Day 19 of lactation. Pups were cross-fostered every 12 h. Two hours
123 following the last vehicle injection, rat dams were deeply anaesthetized with sodium
124 pentobaritone (300 mg/kg) and transcardially perfused with 50 ml of ice-cold saline
125 followed by 250 ml of 4 % paraformaldehyde in 0.1M phosphate buffer (pH 7.4). A
126 second group of lactating rats (bromocriptine plus suckling, n=6) received 500 µg of
127 bromocriptine (500 µg/250 µl saline in 10% ethanol) sub-cutaneously at 8 am and 6
128 pm on Days 17 and 18 of lactation and at 8 am on Day 19 of lactation to suppress
129 production of endogenous prolactin. Pups were cross-fostered every 12 h to ensure
130 pups were fed, enabling maintenance of the suckling-stimulus despite inhibition of
131 milk synthesis in bromocriptine-treated dams. No differences in the number of pups
132 latched to the nipples or the strength of that latching were noted every 12 h when
133 the pups were removed and replaced from either the vehicle- or bromocriptine-
134 treated dams every 12 h. In between cross-fostering time points, no behavioural
135 changes between the pups suckling the bromocriptine-treated dams and those not
136 treated with bromocriptine were noted. Rats were perfused 2 h following the final

bromocriptine injection. A third group of rats (vehicle and no suckling, n=4) received vehicle injections as above on lactation days 17 and 18 and 19 but on day 17 of lactation the pups were removed. Rats were perfused 2 h after the final vehicle injection on day 19.

Finally, to examine the acute response to prolactin, a group of lactating rats (n=9) were injected with bromocriptine at 8 am on day 19 of lactation only as described above. Four hours later these same animals were injected intra-peritoneally with either 1 mg/kg body weight ovine prolactin (n=6; Sigma, St. Louis MO) dissolved in sterile saline or given vehicle alone (n=3). Rats were anaesthetised 45 min later and perfused as described above. Sections from these rats were processed to determine the number of MCH-immunopositive cells that were also immunopositive for pSTAT5.

Following transcardial perfusion, all brains were removed, post-fixed overnight in the same fixative solution, then cryoprotected in a 30% sucrose solution in 0.1 M phosphate buffer till the brains sank. Brains were then frozen on powdered dry ice and stored at -80°C until sectioned using a cryostat. A series of coronal sections (alternatively, 16 µm thick for *in situ* hybridization and 30 µm thick for free-floating immunohistochemistry) were cut through the mPOA from approximately bregma -0.24 mm to bregma -1.32 mm. An additional series of 16 µm and 30 µm thick sections were cut at the level of the incerto-hypothalamic and lateral hypothalamic areas (approximately bregma -1.30 mm to bregma -3.24 mm). Sections for *in situ*

hybridization were mounted onto APS-coated slides and stored at -20°C with dessicant. Sections for immunohistochemistry were collected into cryoprotectant solution in 12-well plates and stored at -20°C.

Double-label in situ *hybridization for Pmch and the long form of the prolactin receptor (Prlr) mRNA*

Preliminary single label *in situ* hybridization experiments comparing the distribution and abundance of *Pmch*-mRNA expressing cells detected using isotopically labelled probes with the distribution detected using non-radioactively labelled probes (digoxigenin), established that with our protocol digoxigenin-labelled probes were sufficiently sensitive and specific to label *Pmch* mRNA in the mPOA. To simultaneously detect mRNA for both *Pmch* and the long form of the prolactin receptor (*Prlr*) in tissue sections, double-label *in situ* hybridizations were performed. Template cDNA was prepared by PCR using primer pairs designed from GenBank (Bethesda, MD) mRNA sequences for pro-melanin-concentrating hormone (*Pmch*: Accession number NM-012625.1, nucleotides 294-527) and the long form of the prolactin receptor (*Prlr*: Accession number NM_001034111, nucleotides 1344-1644). T7 and SP6 promotor sequences were incorporated onto the ends of the primer sequences and the resulting cDNA templates then used to directly transcribe RNA hybridization probes. The specificities of the cDNA templates were confirmed by Sanger sequencing. Antisense and sense probes were synthesized using a digoxigenin RNA-labeling kit for *Pmch* (Roche Diagnostics GmbH, Mannheim) and antisense and sense probes labelled with ^{35}S-UTP were generated for *Prlr* using an *in*

183 *vitro* transcription kit (Promega, Madison, WI). Unincorporated nucleotides were
184 removed by running the probes through mini Quick spin RNA columns (Roche
185 Diagnostics GmbH, Mannheim).

186

187 Sections containing either the mPOA or the incerto-hypothalamic and lateral
188 hypothalamic areas from lactating animals either treated with bromocriptine or
189 vehicle on Days 17, 18 and 19 were thawed for 5 min at 55°C, then immersed in 2 %
190 paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 5 min, washed in sodium
191 citrate buffer (SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0), then permeablized
192 with proteinase K (2 µg/ml) followed by acetylation with 0.1M triethanolamine HCl
193 (pH 8) containing 0.25% acetic anhydride for 10 min. Following washes with SSC,
194 sections were subjected to a series of graded alcohol/chloroform steps, before being
195 air-dried for 2-3 h. A total volume of 90 µl hybridization buffer (100 mM DDT, 0.3 M
196 NaCl, 20 mM Tris pH 8, 5 mM EDTA, 1 X Denhardt's solution, 10 % dextran sulphate,
197 50 % formamide) containing 25 ng of digoxigenin-labeled *Pmch* antisense or sense
198 probe/100 µl of hybridization buffer and approximately 833,300 cpm of ^{35}S-labelled
199 *Prlr* antisense or sense probes/100 µl of hybridization buffer were applied to each
200 slide and the slides coverslipped with Hybrislips.

201

202 Hybridizations were carried out overnight at 55°C then slides were washed with SCC
203 buffer (all post-hybridization SSC washes also had 10 mM B-mercaptoethanol and 1
204 mM EDTA added to the solution) and treated with Ribonuclease A (20 µg/l) for 30

min at room. Following additional SSC washes (most stringent wash, 0.1 X SSC at 64 °C for 2 h), sections were washed in a solution of 100 mM Tris/150mM NaCl (pH 7.5) and then incubated for 48 h with anti-digoxigenin antibody conjugated to alkaline phosphatase (diluted 1:2000). Sections were washed 3 times, then incubated in levamisole (1 mg/ml) solution. The digoxigenin-labeled probes were detected by incubation with NBT/BCIP (nitroblue tetrazolium chloride/5-bromo-4 chloro-3-indolyl-phosphate) substrate for 24 h followed by four, consecutive 30 min washes in buffer (150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA) to eliminate residual NBT and BCIP. Sections were then dipped briefly in distilled water followed by 70% ethanol and dried at RT. Slides were exposed to scientific imaging film for 7 days to generate autoradiograms and subsequently coated with LM-1 Hypercoat emulsion (Amersham Biosciences), placed in light-proof slide boxes containing desiccant and stored at 4°C for 5 weeks. Slides were developed in Kodak D19, fixed with Ilford Hypan, dehydrated through graded ethanols and cleared in xylene before coverslipping with VectaMount™ mounting medium.

Double-label immunohistochemistry for MCH and pSTAT5

Sections taken from the mPOA and the incerto-hypothalamic and lateral hypothalamic areas of the lactating animals given bromocriptine and prolactin or vehicle on Day 19 only were dual-labelled for MCH and pSTAT5. Sections were treated as for single-label immunohistochemistry (described below) with the addition of an antigen-retrieval step (for pSTAT5: 27): sections were incubated with 0.01M Tris (pH10) for 10 min at 90°C immediately following 6 x washes in 0.05M TBS

to remove cryoprotectant. Sections were then incubated in blocking solution (0.05 M TBS, 0.25% Triton-X-100, 2 % BSA) for 1 h and endogenous peroxidases quenched in a 1% hydrogen peroxide/ 40% methanol solution for 10 min. After 3 x 10 min washes the tissue was incubated for 48 h at 4 °C in blocking solution containing 1.5% normal goat serum and rabbit anti-pSTAT5 (Tyr694, Cell Signalling Technology, Beverly, MA; RRID:AB_2315225) at a 1:2,000 dilution. After further rinses, and incubation in biotinylated secondary goat anti-rabbit antibody (Vector, BA-1000; RRID:AB_2313606) diluted 1:500 for 90 min at RT, sections were incubated for 1 h at RT in avidin-biotin-complex solution (ABC Elite kit; Vector Laboratories, Burlingame, CA, USA). Staining for pSTAT5 was then visualized using nickel-enhanced 3-3'-diaminobenzidine solution catalyzed with glucose oxidase to produce black nuclear staining. This step was followed by a second peroxidase activity quenching step before incubating tissue in blocking solution containing 1.5% normal goat serum and anti-MCH (M8440, Sigma-Aldrich; RRID:AB_260690) diluted 1:80,000 for 24-48 h at 4°C. After a TBS wash, sections were incubated with secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG (DAKO) at 1:400 dilution for 90 min. Sections were then reacted with non-nickel-enhanced 3-3'-diaminobenzidine solution resulting in brown cytoplasmic staining of MCH-immunopositive cells. Sections were mounted onto slides and processed as for single label immunohistochemistry (below). In addition, each run included negative control sections in which the primary antibody was omitted from the wells. No staining was observed in these sections.

Single-label immunohistochemistry for MCH

To identify the number of MCH-immunopositive cells in the mPOA following manipulation of prolactin release, free-floating sections containing either the mPOA or the incerto-hypothalamic and lateral hypothalamic area were washed 6 x in 0.05 M TBS (50 mM Tris, 150 mM NaCl, pH 7.6) to remove cryoprotectant, followed by incubation in blocking solution (0.05 M TBS, 0.25% Triton-X-100, 2 % BSA) for 1 h. Endogenous peroxidases were quenched in a 1% hydrogen peroxide/ 40% methanol solution for 10 mins, washed again then incubated for 48 h at 4°C in blocking solution containing 1.5% normal goat serum and anti-MCH (M8440, Sigma-Aldrich; RRID:AB_260690) at a 1:80,000 dilution. Following further washes in 0.05M TBS, the tissue was incubated for 2-3 h with biotinylated secondary goat anti-rabbit antibody diluted 1:500. Next, sections were incubated with avidin-biotin-complex (ABC Elite kit; Vector Laboratories, Burlingame, CA, USA) for 1 h at RT. Finally, MCH immunoreactively was visualized by incubation in nickel-enhanced 3-3'-diaminobenzidine solution catalyzed with glucose oxidase to produce black cytoplasmic staining. Sections were then mounted onto gelatin-coated slides, dried overnight before dehydration in a series of graded alcohols, cleared in xylene and coverslipped under DPX-mounting medium.

Analyses

The total number of *Pmch* mRNA-expressing cells and the number of *Pmch* mRNA-expressing cells that co-expressed *Prlr* mRNA within the mPOA on both sides of the

third ventricle were analysed in two sections per animal (n=4 vehicle-treated animals; n=4 bromocriptine-treated animals). Sections were photographed under brightfield illumination at 400 x magnification. *Pmch* mRNA-expressing cells were identified by the presence of purple/red cytoplasmic staining. To identify double-labelled cells, the number of silver grains (representing *Prlr* mRNA) overlying each *Pmch* mRNA-expressing cell within the mPOA, was quantified using the particle counting function of ImageJ software. For each section, five background measurements of silver grain densities also were made over adjacent areas of the section. Cells were considered positively labeled for *Prlr* mRNA if the signal to background ratio was greater than 3 times mean background values. In sections of the incerto-hypothalamic and lateral hypothalamic areas, no cells were double-labelled for *Pmch* mRNA and *Prlr* mRNA, therefore, cell numbers were not quantified in these areas.

For the analysis of co-localization of MCH-immunopositive cells activated by pSTAT5, digital images were captured under brightfield at 200 X magnification using an Olympus AX70 microscope and QImaging Micropublisher digital camera. The total number of MCH-immunopositive cells and the total number of MCH-immunopositive cells displaying clear nuclear pSTAT5 staining were counted in 3 sections (systematically selected since the anatomical distribution of the MCH-immunopositive cells is very consistent and first cells appear at the same rostro-caudal level) of the mPOA per animal (n=6 prolactin-treated animals; n=3 vehicle-treated animals). The region of the mPOA was outlined and the area overlaid with a

grid using ImageJ. The number of single-labelled and double-labelled cells within each square of the grid that fell within the area of the mPOA were then identified, cateogorized and counted. Only cells that clearly contained brown cytoplasm (MCH-positive cells), or both brown cytoplasmic staining and a distinct black nucleus (double-labelled cells) were counted. Only the brightness and contrast of the images were adjusted. As there was no evidence of double-labelled MCH- and pSTAT5-immunopositive cells in the incerto-hypothalamic and lateral hypothalamic areas sections, cell numbers were not quantified in these sections. Data are presented as the mean percentage of double labelled cells of all the MCH-immunopositive cells per rat. Experimental differences were analysed by Student's t-test.

For the analysis of the single-label immmunohistochemistry, sections were imaged as for dual-label immunohistochemistry. The total number of MCH-immunopositive cells present within the boundaries of the mPOA on both sides of the third ventricle of 3-4 sections per animal were counted. Data are presented as mean number of immunopositive cells counted per rat ± SEM. Differences between groups were analysed by one way analysis of variance followed by Tukey-Kramer *post-hoc* analysis.

All analyses were performed using GraphPad Prism Software and differences between groups were considered significant if $p < 0.05$.

Results

Prepro-MCH (*Pmch*) mRNA was detected in cells within the medial part of the mPOA, as well as in a few cells located more medially to these within the periventricular nucleus, of the hypothalamus of the rat dam on day 19 of lactation (Figure 1a). Double-label *in situ* hybridization for both *Pmch* and *Prlr* mRNA demonstrated co-localization of the two transcripts in some but not all *Pmch* mRNA-positive cells (Figure 1b). When rats were administered with a dopaminergic agonist, bromocriptine, on Days 17, 18 and 19, the number of *Pmch* mRNA-positive cells on Day 19 was markedly decreased compared to vehicle-treated animals (Figure 1c; $p < 0.05$). In both vehicle- and bromocriptine–treated animals, although the total number of positive cells was different, the proportion of *Pmch* mRNA positive cells co-labelled with *Prlr* mRNA was similar (Figure 1d: 61% of vehicle-treated group *versus* 60% of bromocriptine-treated group). Within the medial mPOA, there were also many cells that were *Prlr* mRNA-positive but negative for *Pmch* mRNA: it was however not possible to robustly quantify these cells. Many cells in sections containing the incerto-hypothalamic and lateral hypothalamic areas strongly expressed mRNA for *Pmch* but no co-expression with *Prlr* mRNA was observed (see Figure 1e). No positive labelling was detected in negative control sections incubated with sense probes (data not shown).

To determine if the MCH-immunopositive cells found in the mPOA on Day 19 of lactation were acutely responsive to prolactin, a group of lactating rats were treated with bromocriptine on the morning of Day 19 only. Four hours later these rats

received either prolactin or vehicle and 45 min later were killed. By dual-label immunohistochemistry, sections from these animals were stained for both MCH (DAB: brown cytoplasmic staining) and pSTAT5 (nickel-enhanced DAB: black nuclear staining). Within the medial part of the mPOA, cells were identified that were either MCH-immunopositive, both MCH and pSTAT5 immunopositive as well as pSTAT5 immunopositive alone (Figure 2a, b and c). Of all the MCH-immunopositive cells, the majority (87.3 %) were also immunopositive for pSTAT5 after the animals were treated with prolactin (Figure 2d). When animals were treated with vehicle, the identification of both MCH- and pSTAT5-immunopositive cells was low because of their rarity within the mPOA (Figure 2d). Within the incerto-hypothalamic and the lateral hypothalamic areas, no cells were identified that were both MCH- and pSTAT5-immunopositive (Figure 2e and f).

MCH-immunopositive cells were found in the mPOA on Day 19 of lactation (Figure 3a) and the distribution of the immunopositive cells was very similar to that of cells expressing *Pmch* (Figure 1a). Immunopositive staining for MCH was confined to the cytoplasm of cells (Figure 3a). In the absence of prolactin, induced either by administering bromocriptine on Days 17, 18 and 19 or by withdrawing pups on Day 17, the number of immunopositive cells was markedly reduced on Day 19 (Figure 3b, c and d: 1 way ANOVA, $p < 0.05$).

Discussion

By both *in situ* hybridization and immunohistochemistry, we have confirmed the induction of MCH expression in a specific population of cells within the medial part of the mPOA in established lactation. For the first time we have demonstrated that the majority of these MCH-positive cells also express the long form of the prolactin receptor, and are prolactin responsive during lactation, as indicated by prolactin-induced expression of pSTAT5. Finally, we have shown that suppression of prolactin secretion during established lactation markedly reduced expression of MCH, even in the presence of the ongoing suckling stimulus. These data demonstrate that expression of MCH in the medial part of the mPOA during lactation is dependent on prolactin action.

As others before, we have confirmed that some cells within the medial part of the mPOA express *Pmch*/MCH on day 19 of lactation (3, 4, 5, 6). We hypothesized that this transient expression of MCH in the mPOA in late lactation is regulated by prolactin. To exert its biological effects, prolactin binds to the long form of the prolactin receptor (*Prlr*). Expression of the long form of the receptor has been previously demonstrated in the mPOA of both pregnant and early lactating rats (18, 22, 28). Using double *in situ* hybridization, we have now demonstrated that the long form of the Prlr is also expressed in the mPOA of the late lactating rat and that a sub-population of these *Prlr* mRNA-positive cells also express *Pmch*.

When prolactin binds to the long form of the Prlr the JAK/STAT intracellular signalling pathway is activated and as a result STAT5 is phosphorylated. Phosphorylated STAT5 (pSTAT5) acts a transcription factor to elicit the biological effects of prolactin (29). The detection of immunopositive pSTAT5 is used as a surrogate marker of the long form of the Prlr because the available antibodies to the long form of the Prlr are often not sensitive enough (30). Low numbers of cells immunopositive for pSTAT5 have previously been detected in the mPOA of virgin mice with the number increasing in both pregnant and early lactating mice (27, 31). We have now demonstrated that a significant proportion of the immunopositive MCH cells found in the mPOA are also immunopositive for pSTAT5 in response to prolactin administration, but not vehicle, four hours after treatment with bromocriptine on the morning of Day 19 of lactation only. Hence, in late lactation a high proportion of the transiently-expressing MCH cells in the mPOA are being activated by prolactin.

The pattern and number of *Pmch*/ MCH-immunopositive cells has been studied during lactation in both the incerto-hypothalamus and lateral hypothalamic areas, by several groups, but usually as a single entity rather than as two distinct areas. It has been reported as being decreased (32), unchanged (3, 4, 5) or increased by lactation (33). In our study we noted no alteration in pattern of MCH-immunopositive cells in the incerto-hypothalamus and found no MCH-immunopositive cells co-localized with pSTAT5 suggesting that prolactin had not acted on any of these cells.

Expression of MCH in cells within the medial part of the mPOA in late lactation requires prolactin, but not suckling *per se*, and we have demonstrated this in two ways. On Day 19 of lactation, the number of MCH-immunopositive cells was reduced after prolactin release was inhibited either by treatment with bromocriptine on Days 17, 18 and 19 or pup withdrawal on Day 17 compared with the vehicle treated controls; that is, with or without the maintenance of a suckling stimulus, respectively. We have demonstrated that these MCH-immunopositive cells are also pSTAT5 immunopositive and hence prolactin activated. Others have demonstrated that the number of suckling pups in late lactation determines the number of cell bodies within the medial part of the mPOA expressing MCH (6). This would suggest that neuronal stimulation may stimulate transient MCH expression however it is likely that this is an indirect effect. Alvisi and colleagues (5) found no co-localization of suckling-induced c-Fos expression with MCH in the medial part of the mPOA on days 15 to 21 of lactation and central administration of prolactin does not induce c-Fos expression in the mPOA of rats (24). There were still some cells in the mPOA transiently expressing MCH after the bromocriptine treatment or pup withdrawal suggesting that either a yet to be identified factor is also involved in their maintenance or that the time period of the withdrawal of maintenance was insufficient to extinguish all expression. Based on our data it would appear therefore that the suckling stimulus *per se* is not maintaining the expression of the MCH but rather that it is the suckling-induced release of prolactin that is responsible (25).

Although we have demonstrated that prolactin is involved in the maintenance of these transiently expressing MCH cells within the mPOA in late lactation, it is unlikely that prolactin initiates this transient expression. Others have reported that the transiently expressing MCH cell bodies first appear on Days 8 to 12 of lactation (3, 4). At this time circulating concentrations of prolactin, albeit not as high as in the early days of lactation, are still very high compared to a non-lactating female (26). It is possible that progesterone may be the primary initiating signal. The secretion of progesterone from the corpora lutea of lactation has reached a maximum by Day 8 and started to decline at Day 12 (35). There are progesterone receptors in the mPOA at this time: one group reports that the number of oestrogen-induced progesterone receptors in the mPOA appear to increase in response to increased circulating oestrogen concentrations in late lactation (day 15 compared to day 20 post-partum) (36) whilst another reports that the number of progesterone receptors in the mPOA appears to have stabilized at pre-pregnancy numbers by Day 7 of lactation (34). Progesterone induces changes in maternal behaviour in the second half of lactation (34) and these effects may be mediated by the transiently expressing MCH cells since administration of MCH into the mPOA in early lactation suppresses maternal behaviours (48). Another possible trigger may be the mid-lactation decrease in circulating leptin concentrations (37, 38, 39; reviewed by 40). Leptin receptors have been detected in the mPOA of female non-pregnant, non-lactating mice and rats (41, 42). In the *ob/ob* mouse, MCH expression within the incerto-hypothalamic and lateral hypothalamic areas is increased (43) hence the mid-lactation nadir in leptin may be responsible for up-regulating MCH expression in the medial part of the mPOA and then prolactin maintains expression. It is also

possible that another as yet unidentified trigger could be responsible for initiating the transient expression of MCH in these cells within the mPOA hence further research is required.

The role of the MCH-expressing cells within the medial part of the mPOA remains uncertain, and we have not directly addressed this in the present study. As they are maintained by prolactin, then it can be assumed that they are mediating some of the known roles of prolactin in late lactation. In late lactation, the possible roles for the transient prolactin-induced expression of MCH in the mPOA fall broadly into three areas: adaptive reduction of maternal behaviour preceding weaning; regulation of the dam's return to reproductive activity; and modulation of maternal energy homeostasis.

A role for MCH in stimulating maternal behaviours (nest building, pup retrieval and maternal aggression) in early lactation has been suggested (44, 45), but such effects precede the transient expression of MCH in the medial part of the mPOA and are likely to involve incerto-hypothalamic MCH neurones. In late lactation, the dam's behaviour to her pups changes as she begins to encourage their increasing independence as weaning approaches. These changes in behaviour include a reduction in pup retrieving and nest building (14) as well as a reduction in the dam's aggression in response to intrusion by strange male conspecifics (46, 47). In contrast to the stimulatory actions of incerto-hypothalamic MCH neurones, MCH administration into the mPOA of dams inhibits the normal pup retrieval and nest

476 building behaviours of early lactation, hence mimicking the behaviours seen in late
477 lactation (48). Prolactin is thought to be involved in the onset of maternal behavior
478 at birth, but is not required for ongoing expression of maternal behaviour. Whether
479 there is an active role for prolactin, through its action on the transient MCH-
480 expressing cells of the medial mPOA, in termination of maternal behavior during late
481 lactation has not been evaluated.
482

483 It has long been proposed that in late lactation, lactational anoestrus is primarily
484 maintained by prolactin (49). The mPOA is a site of kisspeptin and GnRH cell bodies
485 that are known to be activated as part of the LH surge (50, 51, 52). Bilateral
486 administration of MCH into the mPOA of ovariectomized, oestradiol benzoate-
487 primed rats results in the generation of LH surge-like release of LH (53, 54, 55). It is
488 therefore possible that the transiently expressing MCH cells are involved in
489 stimulating the LH surge that occurs on Day 20 post-partum in the absence of post-
490 partum mating and in the presence of lactation (16). As for the maternal behaviour
491 effect, it is difficult though to conceive a role for prolactin in the regulation of the
492 dam's return to reproductive activity given the well established inhibitory effects of
493 prolactin on the hypothalamic-pituitary-gonadal axis. Recent data suggests that the
494 inhibitory actions of hyperprolactinemia on the hypothalamic-pituitary-gonadal axis
495 are exerted through the kisspeptin neurones of the arcuate (56) and therefore
496 further work is required to understand prolactin's role(s) in other hypothalamic
497 areas.

498

Both incerto-hypothalamic and lateral hypothalamic MCH play a significant role in stimulating food intake (43) and MCH expression in these areas are increased by fasting (57). It is possible that increased MCH in the mPOA during lactation could contribute to this orexigenic role. Towards the end of the lactation although the animals are not fasting, *per se,* they are in a state of negative energy balance as they utilize body reserves to maintain milk production. During the second half of lactation food consumption is higher (15, 58) and this appears to be regulated by prolactin, as bromocriptine treatment will reduce food intake while bromocriptine plus chronic intraventricular infusion of prolactin will restore food consumption to control amounts (59). The prolactin-activated MCH cell bodies in the medial part of the mPOA may have a role in increasing food intake. As suggested by Rondini and colleagues, it is also possible that other neuropeptides related to metabolic control and/or leptin may be responsible for the increased expression of MCH in the mPOA (4); that is, that the role of these transiently expressing MCH, prolactin activated cells in the face of the "increased energy drain" of late lactation is to stimulate feeding above the already increased levels seen earlier in lactation. Further research is required to determine if these transiently expressing MCH cell bodies have projections that extend to the third ventricle and participate in the recently described stimulation of feeding behaviour by MCH through cerebral ventricular volume transmission (60).

We have demonstrated that the majority of MCH-immunopositive cells that are transiently expressed in the medial part of the mPOA during late lactation express

the prolactin receptor and that the maintenance of MCH expression in these cells is dependent on prolactin action. Further research is required to determine the functional role(s) of these prolactin-activated transiently MCH-expressing cells however the most likely role would appear to involve adaptations in maternal metabolism to support the final week of lactation.

Acknowledgements

We thank Tony Sapsford and Amanda Wyatt for their excellent technical assistance. This work was supported by an HRC Programme Grant 14/568 (DRG), a Society for Reproduction and Fertility Academic Scholarship (JFM) and a British Society for Neuroendocrinology Research Visit Grant (JFM).

Figure Legends

Figure 1. *In situ* hybridization showing expression of *Pmch* mRNA and *Prlr* mRNA expressing neurones in sections from a rat dam treated with vehicle (on Days 17, 18 and 19) and suckling pups on Day 19 of lactation (panels a, b and f) and a rat dam treated with bromocriptine (on Days 17, 18 and 19) and suckling pups on Day 19 of lactation (panel c): a. Low power dark-field photomicrograph showing the distribution of *Pmch* mRNA (white clusters of silver grains) in the mPOA; b. representative brightfield image of *Pmch* mRNA-expressing cells (purple/red cytoplasmic labeling) dual-labeled for prolactin receptor (*Prlr*) mRNA (black clusters of silver grains over cells). Top insert shows a high power image of the area outlined in the black box of a cell expressing *Pmch* mRNA only. Bottom insert shows a high power image of the area outlined in the black box of two positive dual–labelled cells; c. representative brightfield image of *Pmch* mRNA-expressing cells (purple/red cytoplasmic labeling) dual-labeled for prolactin receptor (*Prlr*) mRNA (black clusters of silver grains over cells). Insert shows a high power image of the area outlined in the black box of a positive dual–labelled cell; d. quantification of the mean (± SEM) total number of *Pmch* mRNA-expressing cells per rat in the mPOA of rat dams suckling pups on day 19 of lactation after receiving either vehicle (n=4 animals) or bromocriptine treatment (n=4 animals). *$p < 0.05$; e. The proportion of *Pmch*-expressing neurones co-expressing *Prlr* mRNA in the mPOA on day 19 of lactation following vehicle or bromocriptine treatment; f. representative low power image of *Pmch* mRNA-expressing cells (purple/red cytoplasmic labeling) dual labelled for *Prlr* mRNA in the incerto-hypothalamic and lateral hypothalamic areas. In these regions,

no *Pmch* mRNA-expressing cells were observed to co-express *Prlr* mRNA. A high power image of two single-labelled *Pmch* mRNA-expressing cells is shown in the insert in e. Abbreviations, f, fornix; mPOA, medial preoptic area; och, optic chiasm; 3V, third ventricle. Scale bars: (a) = 150 µm; (b and c) = 50 µm, inserts in (b and c) = 10 µm; (e) = 100 µm, insert in (e) = 10 µm.

Figure 2. Double label immunohistochemistry for melanin concentrating hormone (MCH: brown cytoplasmic staining) and phosphorylated Stat5 (pStat5: black nuclear staining) following prolactin administration, in the medial preoptic area (mPOA) of dams on Day 19 of lactation: a. representative image of the mPOA (scale bar: 50 µm); b. and c. higher magnification of the boxed areas in (a) with examples of cells either immuno-positive for MCH (blue arrow) or pSTAT5 alone (orange arrow) or immuno-positive for both MCH and pSTAT5 (black arrow) (scale bar: 15 µm); d. quantification of the percentage (± SEM) of cell bodies in the mPOA that were co-labelled for MCH and pSTAT5 following administration of either prolactin or vehicle. *$p < 0.05$; e. and f. representative images of (e) low and (f) high, magnification of the lateral hypothalamus of a rat dam on Day 19 of lactation following prolactin administration showing MCH immuno-labelling (brown cytoplasmic staining). No MCH-immunopositive cell bodies are also immunopositive for pSTAT5. Arrows indicate neurones single-labelled for MCH. Scale bars: (e) = 100 µm and (f) = 30 µm. Abbreviations, f, fornix; ic, internal capsule.

Figure 3. Representative images of cell bodies immuno-positive for melanin concentrating hormone (MCH) in the medial preoptic area of the rat dam on Day 19

of lactation in animals either with circulating concentrations of prolactin characteristic of lactation (vehicle) or treated to suppress prolactin release (bromocriptine or pup removal) on Days 17, 18 and 19 of lactation. a. Vehicle was administered on Days 17, 18 and 19 of lactation: insert shows examples of cells of interest at 600 fold magnification (indicated by black arrows); b. bromocriptine was administered on the mornings of Days 17, 18 and 19 of lactation; and c. pups were removed on the morning of Day 17 and then the dam received vehicle on Days 17, 18 and 19 of lactation. All animals were perfused on the afternoon of Day 19 and the brains prepared for immunohistochemistry. All low power images taken at 40 x magnification. Scale bars: (a-c) = 500 µm insert and insert in (a) = 5 µm. mPOA, medial preoptic area; och, optic chiasm; 3v, third ventricle. d. Quantification of the number of cell bodies immuno-positive for MCH in the medial preoptic area (mPOA) of the rat dam on Day 19 of lactation in animals either with circulating concentrations of prolactin characteristic of lactation (a. vehicle, n = 6) or treated to suppress prolactin release (b. bromocriptine, n = 6 or c. pup removal, n = 4) on Days 17, 18 and 19 of lactation. Data are presented as the mean ± SEM. Differences between means were analysed by one-way ANOVA followed by Turkey-Kramer test, ($F_{2,13}$ = 18.98): vehicle versus prolactin withdrawal. Bars with different letters are significantly different ($p < 0.05$).

599 **References**

600 1. Naufahu J, Cunliffe AD, Murray JF. The roles of melanin-concentrating hormone in
601 energy balance and reproductive functions: are they connected? Reproduction
602 2013; 146: R141-R150.

603 2. Diniz GB, Bittencourt JC. The melanin-concentrating hormone as an integrative
604 peptide driving motivated behaviors. Front Syst Neurosci 2017; 11: 32.

605 3. Knollema S, Brown ER, Vale W, Sawchenko PE. Novel hypothalamic and preoptic
606 sites of prepro-melanin-concentrating hormone messenger ribonucleic Acid
607 and Peptide expression in lactating rats**.** J Neuroendocrinol 1992; 4: 709-717.

608 4. Rondini TA, Donato Jr J, Rodrigues Bde C, Bittencourt JC, Elias CF. Chemical
609 identity and connections of medial preoptic area neurons expressing melanin-
610 concentrating hormone during lactation. J Chem Neuroanat 2010; 39: 51-62.

611 5. Alvisi R, Diniz GB, Da-Silva JM, Bittencourt JC, Felicio LF. Suckling-induced *Fos*
612 activation and melanin-concentrating hormone immunoreactivity during late
613 lactation. Life Sci 2016; 148: 241-246

614 6. Ferreira JGP, Duarte JCG, Diniz GB, Bittencourt JC. Litter size determines the
615 number of melanin-concentrating hormone neurons in the medial preoptic
616 area of Sprague Dawley lacting dams. Physiol Behav 2017; 181: 75-79.

617 7. Numan M. (1974) Medial preoptic area and maternal behavior in the female rat.
618 J Compar Physiol Psychol 1974; 87: 746-759.

619 8. Terkel J, Bridges RS, Sawyer CH. Effects of transecting lateral neural connections
620 of the medial preoptic area on maternal behavior in the rat: nest building, pup
621 retrieval and prolactin secretion. Brain Res 1979; 169: 369-380.

622 9. Numan M, Callahan EC. (1980) The connections of the medial preoptic region and
623 maternal behaviour in the rat. Physiol Behav 1980; 25: 653-665.

624 10. Dobolyi A, Grattan DR, Stolzenberg DS. Preoptic inputs and mechanisms that
625 regulate maternal responsiveness. J Neuroendocrinol 2014; 26: 627–640.

626 11. Bridges RS. Neuroendocrine regulation of maternal behaviour. Front
627 Neuroendocrinol 2015; 36: 178-196.

628 12. Kohl J, Autry AE, Dulac C. The neurobiology of parenting: A neural circuit
629 perspective. Bioessays 2017; 39: 1-11.

630 13. Brown RSE, Aoki M, Ladyman SR, Phillipps HR, Wyatt A, Boehm U, Grattan DR.
631 Prolactin action in the medial preoptic area is necessary for postpartum
632 maternal nursing behavior. Proc Natl Acad Sci U S A 2017; 114: 10779-10784.

633 14. Pereira M, Morrell JI. Changing role of the medial preoptic area in the regulation
634 of maternal behaviour across the post-partum period: facilitation followed by
635 inhibition. Behav Brain Res 2009; 205: 238-248.

636 15. Ota K, Yokoyama A. Body weight and food consumption of lactating rats: effects
637 of ovariectomy and of arrest and resumption of suckling. J Endocrinol 1967;
638 38: 251-261.

639 16. Woodside B. Effects of food restriction on the length of lactational diestrus in
640 rats. Horm Behav 1991; 25: 70-83.
641 17. Grattan DR, Bridges RS. Prolactin actions in the brain. In: Pfaff DW, Etgen AM,
642 Fahrbach SE, Rubin RT eds. Hormones, brain and behaviour. New York:
643 Academic Press; 2009: 2471-2503.

644 18. Chui S, Wise PM. Prolactin receptor mRNA localization in the hypothalamus by in
645 situ hybridization. J Neuroendocrinol 1994; 6: 191-199.

646 19. Bakowska JC, Morrell JI. (1997) Atlas of the neurons that express mRNA for the
647 long form of the prolactin receptor in the forebrain of the female rat. J Comp
648 Neurol 1997; 386: 161-177.

649 20. Pi X, Grattan DR. Distribution of prolactin receptor immunoreactivity in the brain
650 of estrogen-treated, ovariectomized rats. J Comp Neurol 1998; 394: 462-474.

651 21. Kokay I, Wyatt A, Phillupps HR, Aoki M, Ectors F, Boehm U, Grattan DR. Analysis
652 of prolactin receptor expression in the murine brain using a novel prolactin
653 receptor reporter mouse. J Neuroendocrinol 2018: 30: e12634

654 22. Pi X, Grattan DR. Increased expression of both short and long forms of prolactin
655 receptor mRNA in hypothalamic nuclei of lactating rats. J Mol Endocrinol 1999;
656 23: 13-22.

657 23. Pi X, Grattan DR. Expression of prolactin receptor mRNA is increased in the
658 preoptic area of lactating rats. Endocrine 1999; 11: 91-98.

659 24. Cave BJ, Wakerley JB, Luckman SM, Tortonese DJ. Hypothalamic targets for
660 prolactin: assessment of c-Fos induction in tyrosine hydroxylase- and
661 proopiomelanocortin-containing neurones in the rat arcuate nucleus following
662 acute central prolactin administration. Neuroendocrinology 2001; 74: 386-
663 395.

664 25. Grosvenor CE, Mena F, Whitworth NS. The secretion rate of prolactin in the rat
665 during suckling and its metabolic clearance rate after increasing intervals of
666 nonsuckling. Endocrinology 1979; 104: 372-376.

667 26. Grattan DR. The actions of prolactin in the brain during pregnancy and lactation.
668 Prog Brain Res 2001; 133: 153-171.

669 27. Brown RS, Kokay IC, Herbison AE, Grattan DR. Distribution of prolactin-
670 responsive neurons in the mouse forebrain. J Comp Neurol 2010; 518: 92-102.

671 28. Augustine RA, Kokay IC, Andrews ZB, Ladyman SR , Grattan DR. Quantitation of
672 prolactin receptor mRNA in the maternal rat brain during pregnancy and
673 lactation. J Mol Endocrinol 2003: 31: 221-232.

674 29. Bole-feysot C, Goffin V, Edery M, Binart N, Kelly PA. Prolactin (PRL) and its
675 receptor: actions, signal transduction pathways and phenotypes observed in
676 PRL receptor knockout mice. Endocr Rev 1998; 19: 225-268.

677 30. Sapsford TJ, Kokay IC, Ostberg L, Bridges RS, Grattan DR. Differential sensitivity
678 of specific neuronal populations of the rat hypothalamus to prolactin action. J
679 Comp Neurol 2012; 520: 1062-1077.

680 31. Salais-López H, Lanuza E, Agustín-Pavón C, Martínez-García F. Tuning the brain
681 for motherhood: prolactin-like central signalling in virgin, pregnant, and
682 lactating female mice. Brain Struct Funct 2017; 222: 895-921.

683 32. García MC, López M, Gualillo O., Seoane LM, Diéguez C., Señarís RM.
684 Hypothalamic levels of NPY, MCH, and prepro-orexin mRNA during pregnancy
685 and lactation in the rat: role of prolactin. FASEB J 2003; 17: 1392-1400.

686 33. Sun G, Tian Z, Murata T, Narita K, Honda K, Higuchi T. Central and peripheral
687 immunoreactivty of melanin-concentrating hormone in hypothalamic obese
688 and lactating rats. J Neuroendocrinol 2004; 16: 79-83.

689 34. Grieb ZA, Tierney SM, Lonstein JS. Postpartum inhibition of ovarian steroid
690 action increases aspects of maternal caregiving and reduces medial preoptic
691 area progesterone receptor expression in female rats**.** Horm Behav 2017; 96:
692 31-41.

693 35. Taya K, Greenwald GS. Peripheral blood and ovarian levels of sex steroids in the
694 lactating rat. Endocrinol Jpn 1982; 29: 453-459.

695 36. Barbosa-Vargas E, Pfaus JG, Woodside B. Sexual behavior in lactating rats: Role
696 of estrogen-induced progesterone receptors. Horm Behav 2009; 56: 246-253.

697 37. Brogan RS, Grove KL, Smith MS. Differential regulation of leptin receptor but not
698 orexin in the hypothalamus of the lactating rat. J Neuroendocrinol 1997; 12:
699 1077-1086.

700 38. Woodside B, Abizaid A, Jafferali S. Effect of acute food deprivation on lactational
701 infertility in rats is reduced by leptin administration. Am J Physiol 1998; 274
702 (Regulatory Integrative Comp Physiol 43): R1653–R1658.
703 39. Woodside B, Abizaid A, Walker CD. (2000) Changes in leptin levels during
704 lactation: implications for lactational hyperphagia and anovulation. Horm
705 Behav 2000; 37: 353-365.

706 40. Woodside B. Mood, food, and fertility: Adaptations of the maternal brain.
707 Compr Physiol 2016; 6: 1493-1518.

708 41. Diano S, Kalra SP, Sakamoto H, Horvath TL. Leptin receptors in estrogen
709 receptor-containing neurons of the female rat hypothalamus. Brain Res 1998;
710 812: 256-259.

711 42. Zhang Y, Kerman IA, Laque A, Nguyen P, Faouzi M, Louis GW, Jones JC, Rhodes C,
712 Münzberg H. Leptin-receptor-expressing neurons in the dorsomedial
713 hypothalamus and median preoptic area regulate sympathetic brown adipose
714 tissue circuits. J Neurosci 2011; 31: 1873-1884.

715 43. Qu D, Ludwig DS, Gammeltoft S, Piper M, Pelleymounter MA, Cullen MJ, Mathes
716 WF, Przypek R, Kanarek R, Maratos-Flier E. A role for melanin-concentrating
717 hormone in the central regulation of feeding behaviour. Nature 1996; 380:
718 243-247.
719 44. Adams AC, Domouzoglou EM, Chee MJ, Segal-Lieberman G, Pissios P, Maratos-
720 Flier E. Ablation of the hypothalamic neuropeptide melanin concentrating

721 hormone is associated with behavioral abnormalities that reflect impaired
722 olfactory integration. Behav Brain Res 2011; 224: 195-200.
723 45. Alachkar A, Alhassen L, Wang Z, Wang L, Onouye K, Sanathara N, Civelli O.
724 Inactivation of the melanin concentrating hormone system impairs maternal
725 behaviour. Eur Neuropsychopharmacol 2016; 26: 1826-1835.
726 46. Flannelly KJ, Flannelly L. Time course of postpartum aggression in rats (Rattus
727 norvegicus). J Comp Psychol 1987; 101: 101-103.
728 47. Mayer AD, Reisbick S, Siegel HI, Rosenblatt JS. Maternal aggression in rats:
729 changes over pregnancy and lactation in a Sprague-Dawley strain. Aggress
730 Behav 1987; 13: 29-43.
731 48. Benedetto L, Pereira M, Ferreira A, Torerolo P. Melanin-concentrating hormone
732 in the medial preoptic area reduces active components of maternal behavior in
733 rats. Peptides 2014; 58: 20-25.
734 49. Hansen S, Sodersten P, Eneroth P. Mechanisms regulating hormone release and
735 the duration of dioestrus in the lactating rat. J Endocrinol 1983; 99: 173-180.
736 50. Lee WS, Smith MS, Hoffman GE. Luteinizing hormone-releasing hormone
737 neurons express Fos protein during the proestrous surge of luteinizing
738 hormone. Proc Natl Acad Sci U S A 1990; 87: 5163-5167.
739 51. Porkka-Heiskanen T, Urban JH, Turek FW, Levine JE. (1994) Gene expression in a
740 subpopulation of luteinizing hormone-releasing hormone (LHRH) neurons prior to
741 the preovulatory gonadotropin surge. J Neurosci 1994; 14: 5548-5558.

742 52. Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyama Y, Iwata K, Yamada S,
743 Inoue K, Ohtaki T, Matsumoto H, Maeda K. (2005) Involvement of central
744 metastin in the regulation of preovulatory luteinizing hormone surge and
745 estrous cyclicity in female rats. Endocrinology 2005; 146: 4431-4436.

746 53. Gonzalez MI, Baker BI, Wilson CA. Stimulatory effect of melanin-concentrating
747 hormone on luteinising hormone release. Neuroendocrinology 1997; 66: 254-
748 262.

749 54. Murray JF, Adan RAH, Walker R, Baker BI, Thody AJ, Nijenhuis WAJ, Yukitake J,
750 Wilson CA. Melanin-concentrating hormone, melanocortin receptors and
751 regulation of LH release. J Neuroendocrinol 2000; 12: 217-223.

752 55. Murray JF, Hahn JD, Kennedy A, Small C, Bloom SR, Haskell-Luevano C, Coen CW,
753 Wilson CA. Evidence for a stimulatory action of melanin concentrating
754 hormone (MCH) on luteinising hormone release involving MCH1 and
755 melanocortin-5 (MC-5) receptors. J Neuroendocrinol 2006; 18: 157-167.

756 56. Brown RSE, Khant Aung Z, Phillipps HR, Barad Z, Lein HJ, Boehm U, Szawka RE,
757 Grattan DR. Acute suppression of LH secretion by prolactin in female mice is
758 mediated by kisspeptin neurons in the arcuate nucleus. Endocrinology 2019;
759 160: 1323–1332.

760 57. Silva JP, von Meyenn F, Howell J, Thorens B, Wolfrum C, Stoffel M. Regulation of
761 adaptive behaviour during fasting by hypothalamic FOXa2. Nature 2009; 462:
762 646-650.

763 58. Cripps AW, Williams VJ. The effect of pregnancy and lactation on food intake,
764 gastrointestinal anatomy and the absorptive capacity of the small intestine in
765 the albino rat. Br J Nutr 1975; 33: 17-32

766 59. Woodside B. Prolactin and the hyperphagia of lactation. Physiol Behav 2007; 91:
767 375-382.

768 60. Nobel EE, Hahn JD, Konanur VR, Hsu TM, Page SJ, Cortella AM, Liu CM, Song MY,
769 Suarez AN, Szujewski CC, Rider D, Clarke JE, Darvas M, Appleyard SM, Kanoski
770 SE. Control of feeding behavior by cerebral ventricular volume transmission of
771 melanin-concentrating hormone. Cell Metabolism 2018; 28: 55-68.

772

773

774

775

776

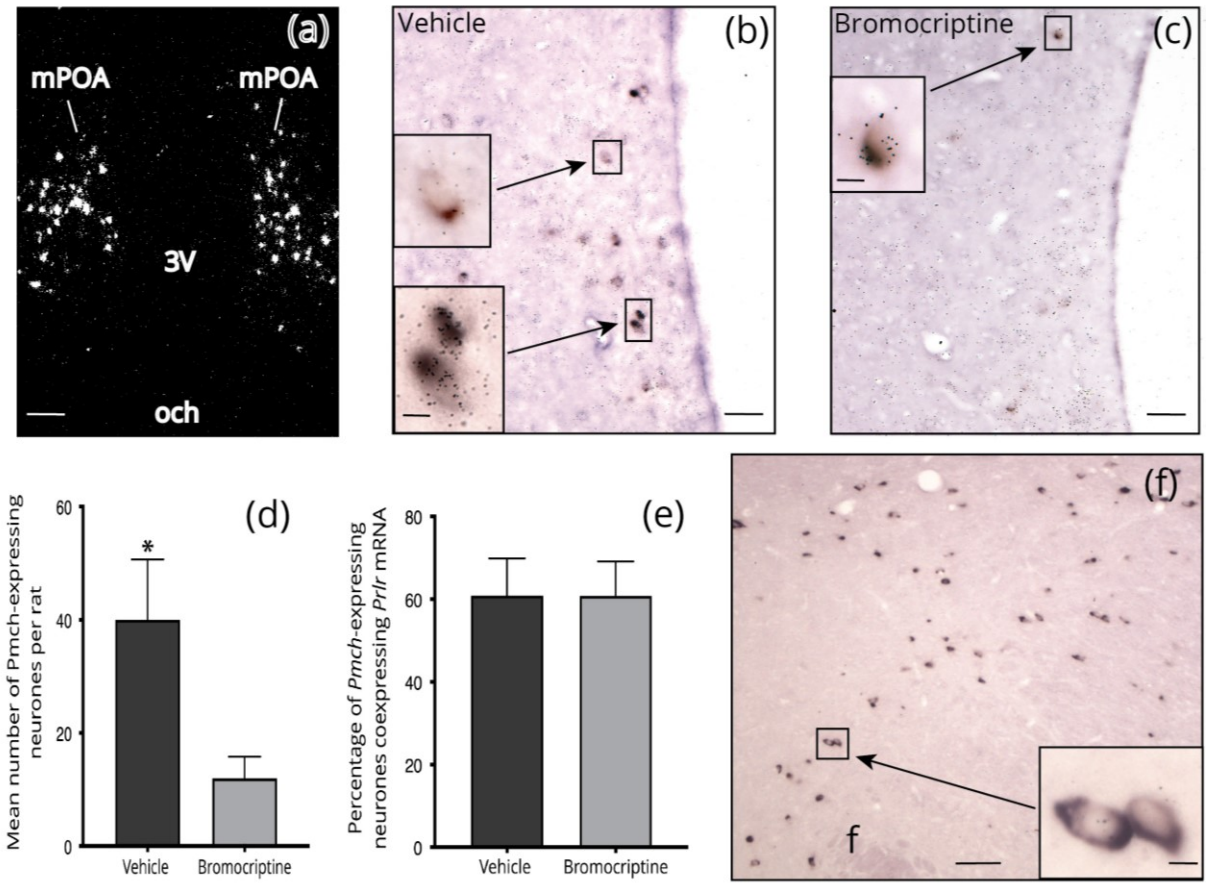
777

778

779

780

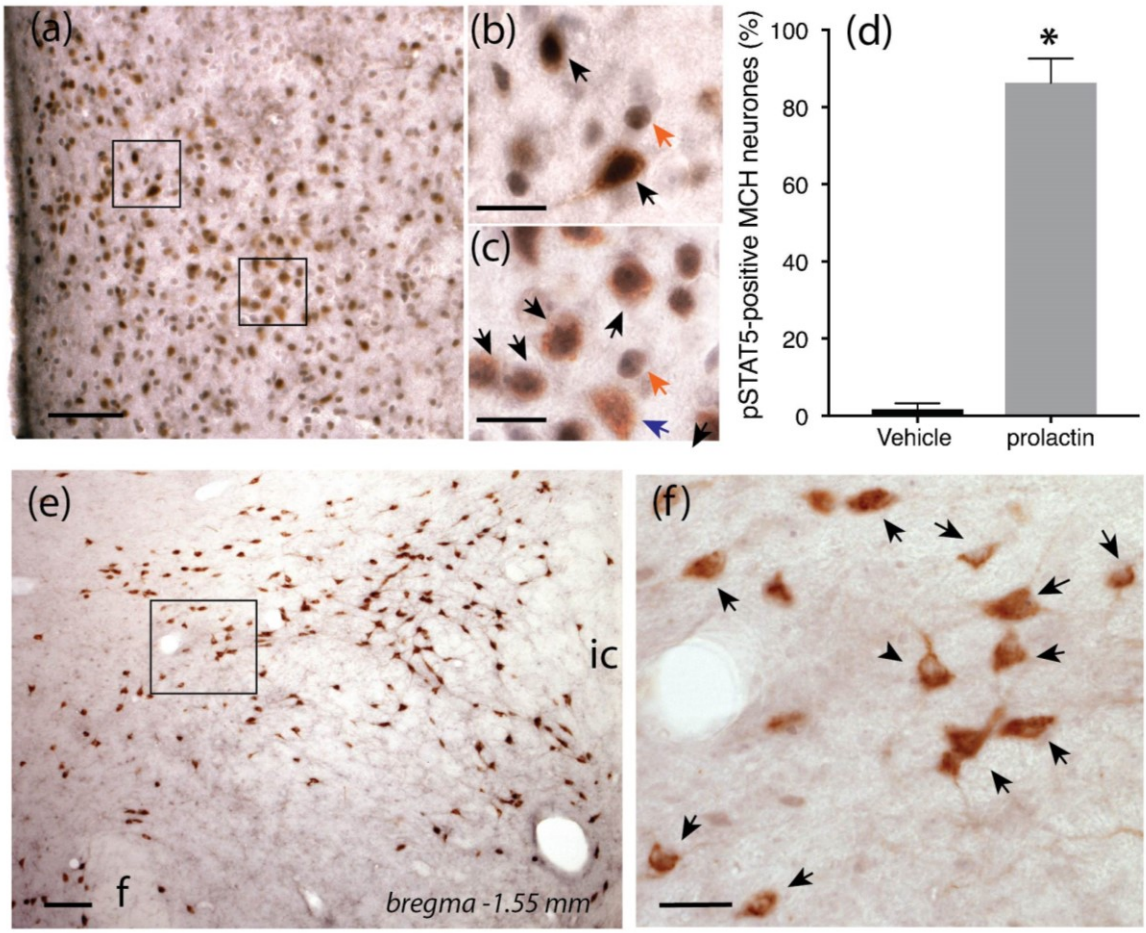
781 Figure 1



782

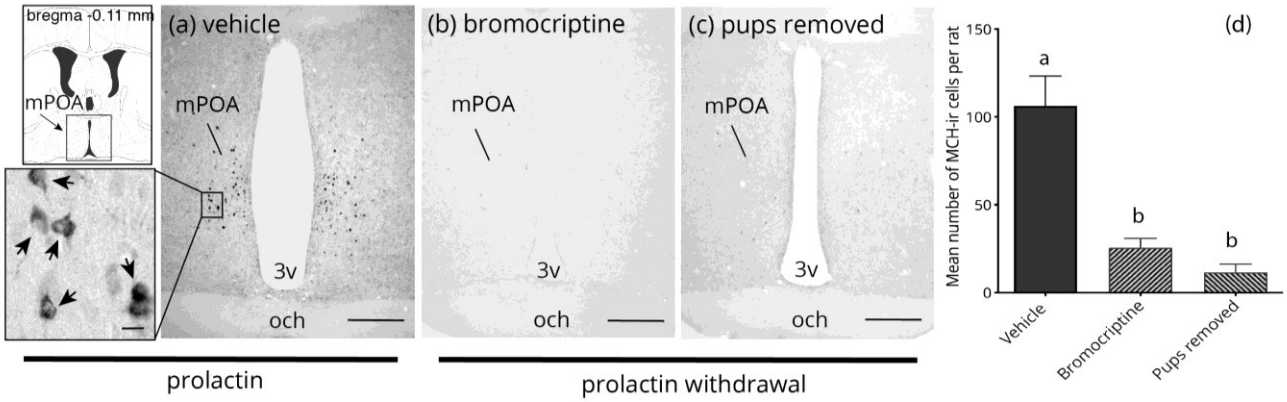
783

784 Figure 2



785

786 Figure 3



787